National Institute of Dental and Craniofacial Research
Division of Intramural Research

Annual Report Summary

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Fiscal Year 2000 Annual Report

National Institute of Dental and Craniofacial Research

Introduction and Overview

Henning Birkedal-Hansen, Scientific Director

INTRODUCTION AND OVERVIEW
2000

The Division of Intramural Research (DIR) witnessed a number of important changes during the Fiscal Year 1999/2000. Dr. Harold Slavkin announced that after 5 years of successfully guiding and directing the National Institute of Dental and Craniofacial Research (NIDCR) he would be leaving to return to his alma mater, the University of Southern California, to serve as Dean of the School of Dentistry. Dr. Slavkin departed in mid-July 2000 and after a national search Dr. Lawrence Tabak was appointed as the seventh NIDCR Director effective on September 1, 2000. Dr. Tabak came to the NIDCR from the University of Rochester where he directed the Center for Oral Biology, Aab Institute of Biomedical Services. Dr. Slavkin provided outstanding leadership to the NIDCR and to the Division of Intramural Research and the scientists and staff of the DIR wish him the best in his new challenges. At the same time the DIR warmly welcomes Dr. Tabak as the new NIDCR Director and looks forward to his leadership in the years ahead.

In another major change, after six highly successful years as the Director of the National Institutes of Health (NIH) Dr. Harold Varmus left in January to serve as president of New York City's Memorial Sloan-Kettering Cancer Center. Until a new NIH Director is appointed, Dr. Ruth Kirschstein is serving as Principal Deputy Director. Under Dr. Varmus' leadership the NIH experienced an extraordinary level of growth and prosperity as evidenced by its level of support for research and by the accelerated construction projects on campus, including the Clinical Research Center, a multipurpose research laboratory building (Building 50) and a Vaccine Research Center (Building 40).

The release of the Surgeon General's report on Oral Health and the emerging focus on health disparities lead the NIDCR to assess its contribution to these important societal and medical/dental problems. As a result, Dr. Tabak's first National Advisory Dental and Craniofacial Research Council meeting in September 2000 was devoted mainly to the topic of health disparities and involved Council members in activities aimed at increasing NIDCR's efforts in this area.

Scientific Productivity

During the past year, the DIR researchers made many significant contributions to science which impact not only on dental and craniofacial research but on biomedical research in general, as evidenced by publication of high profile articles in *Cell, Science, Nature, PNAS* and other leading biomedical research journals. It is impossible to describe all of this research, which spans the spectrum from basic to translational to clinical research in areas related to dental and craniofacial health and disease; however, the highlights are summarized in the Branch reports that follow.

The Division of Intramural Research currently includes 35 independent, senior investigators, 30 who are tenured and 5 who are tenure track. The Senior Investigators and their staff total approximately 360 scientists who are organized into 7 Branches:

- Craniofacial Developmental Biology and Regeneration Branch
- Craniofacial Epidemiology and Genetics Branch
- Craniofacial and Skeletal Diseases Branch
- Gene Therapy and Therapeutics Branch
- Oral Infection and Immunity Branch
- Oral and Pharyngeal Cancer Branch
- Pain and Neurosensory Mechanisms Branch

and four single standing units:

- Functional Genomics Unit
- Immunopathology Section
- Matrix Metalloproteinase Unit
- Molecular Structural Biology Unit

Most of these scientists are located in Building 30 with others working in Buildings 10, 45, 49, or the National Naval Dental Center.

Programmatic Changes

The Board of Scientific Counselors reviewed two Branches and two separate Units during the past year. The Craniofacial Developmental Biology and Regeneration Branch was reviewed in December 1999 together with the Functional Genomics Unit and the Matrix Metalloproteinase Unit. In June of 2000 the Oral and Pharyngeal Cancer Branch was reviewed. Both of these review sessions resulted in the recognition of the high quality and productivity of our programs and yielded constructive suggestions for future research.

The Oral and Pharyngeal Cancer Branch recruited two tenure track scientists over the past year, Dr. Thomas Bugge (mouse geneticist, proteinase function in cancer development) and Dr. Adrian Senderowicz (clinical oncologist). With these recruitments the Branch has greatly expanded its program on head and neck cancer research.

In the past year, Dr. Deborah Winn of the Craniofacial Epidemiology and Genetics Branch accepted a position at the National Cancer Institute. Dr. Winn played a major role in the development of the Surgeon General's Report on Oral Health and spearheaded the Institute's efforts in epidemiology and national surveys. The loss of Dr. Winn's expertise and insight into general as well as dental and craniofacial epidemiology will leave a considerable void and provides an impetus for the DIR, as well as the NIDCR, to consider its future role in epidemiology and in the gathering and analysis of health and disease data.

Dr. E. D. Eanes retired after a long and productive career as a leading expert in mineralization and mineral chemistry. Dr. Eanes' unique expertise will be missed as the Craniofacial and Skeletal Diseases Branch increasingly moves its focus to a translational and clinical direction.

Due to the continuing lack of space on campus, the NIDCR, together with National Institute of Allergy and Infectious Diseases, is planning a joint laboratory research facility in the Twinbrook

area of Rockville, MD. The NIDCR will acquire approximately 10,000 square feet which will provide new space for the expansion of its programs.

Clinical Issues

After assuming the responsibility of Clinical Director, Dr. Raymond A. Dionne quickly proceeded to implement structural as well as programmatic changes in the operation of the Dental Clinic. Dr. Lisa Cayous was recruited as Chief of the Dental Consult Service and since her arrival in April 2000 has done an impressive job of increasing the efficacy and output of the consult service. After Dr. Jane Atkinson, Director, Oral Medicine Training Program, left the NIDCR to pursue new opportunities at the University of Maryland Baltimore College of Dental Surgery, we initiated a detailed analysis of the role, function and efficacy of this and other clinical research training initiatives. Dr. Jaime Brahim was named Acting Director of the program and he is currently working with the Clinical Director and the Office of Education (OEd) to assess the future of the program.

Major clinical initiatives of the past year have focused on expansion of clinical research activities in skeletal diseases, head and neck cancer, AIDS, and pain research. The Division now has approximately 50 active clinical research protocols. The NIDCR's two clinics, the Dental Clinic on the first floor and the Pain Clinic on the 3rd floor, are scheduled for merging and relocation into space on the 13th floor of the ACRF in Building 10. The DIR staff is very much looking forward to the consolidation of all clinical research and training activities into contiguous space.

Research Training

The NIDCR continues to expand its efforts to recruit and train talented individuals to ensure continued enrichment of the oral health research community. The Office of Education (OEd) acts as a focal point for all intramural training programs, particularly those addressing clinical research.

For a number of years the NIDCR has offered two formal (residency) patient-oriented research training programs: the Oral Medicine Research Program and the Residency in Dental Public Health and also participates in the NIH-wide Clinical Research Training Program. The Clinical Research Fellowship is a new program formalized from previous experiences with fellows participating in clinical research training. NIDCR also had its third dental student in the NIH Clinical Research Training Program for Medical and Dental Students. In addition, an NIDCR fellow successfully completed the master's degree program in clinical research in collaboration with Duke University.

The number of training program participants increased this year with the Medical University of South Carolina DMD/PhD collaboration going from one to two summer students; the Residency in Dental Public Health accepting three residents this year, an average increase of two; and, the Clinical Externship program growing from two to 17 students as a direct result of recruiting trips. The new Clinical Research Fellowship has 3 dual-degree dentists receiving their postdoctoral training in clinical research methods.

NIDCR DIR participated in biomedical research fairs and interactive tours of its labs and clinics for minority high school students. In collaboration with the NIDCR Office of Diversity Management, this year NIDCR hosted students from the Meyerhoff Institute, the National Youth Leadership Forum, the National African American Youth Initiative, the National Hispanic Youth Initiative, and the National Native American Youth Initiative.

In addition to participating in the NIH-wide Summer Internship Program for high school, college, and graduate students, this was the fifth year of the NIDCR Summer Dental Student Training Award. This program is designed to promote the professional careers of talented dental students by providing them with early exposure to the latest advances in oral health research. An interim evaluation of the program indicates that the program has been fully successful in reaching its short-term goals at the five-year mark.

Building Renovation and Infrastructure Improvements

During the past year, the DIR undertook an unprecedented number of renovations to Building 10 and three floors of Building 30. Considering the scarcity of swing space, the logistics of the renovation has been a true challenge which has been superbly orchestrated by Dr. Jack London, the Assistant Scientific Director. One of the most limiting factors for expansion of our research program is lack of animal space. Currently, we are examining the possibility of consolidating and expanding the animal facilities in the lower level of Building 30, complete with an independent air handling system and security/access control system. This would allow considerable expansion of the animal facilities as well as resolving a number of problems pertaining to the current animal space and its infrastructure. Subsequently all administrative functions would be consolidated on the renovated 5th floor.

As we look forward to another productive year, we hope that you will take time to review the scientific accomplishments that are reported in the following summaries.

Craniofacial Developmental Biology and Regeneration Branch

Kenneth Yamada
Hynda Kleinman
Yoshihiko Yamada

CRANIOFACIAL DEVELOPMENTAL BIOLOGY AND REGENERATION BRANCH
2000

The Craniofacial Developmental Biology and Regeneration Branch (CDBRB) focuses on creating new research breakthroughs to (a) understand the mechanisms of normal and abnormal craniofacial development and function at genetic, molecular, and cell biological levels, (b) discover new genes, biologicals, and biomimetics relevant to diagnosis, repair, and therapy, and (c) develop creative, biologically based methods to replace craniofacial and other tissues that are defective or damaged. Particular emphasis is placed on the interface between cells and extracellular molecules. Our mission spans the range from basic research to clinical, and from normal development to anomalies, wound healing, cancer, and AIDS. CDBRB researchers are exploring important fundamental questions in developmental biology and related fields, such as the molecular and cell biological mechanisms of morphogenesis, formation and functions of extracellular matrix and its receptors, signaling from the cell surface to the nucleus for novel gene induction, cellular differentiation, wound repair, and cancer cell growth and metastasis. Discoveries and ongoing innovations in this basic research will provide the basis for novel translational and patient-oriented applications. This past year, our researchers continued to generate a variety of exciting research advances and to receive international recognition. We also continued to place high priority on training younger scientists to become independent leaders in academia and industry. In addition, we provided extensive service and citizenship activities on behalf of NIDCR, NIH, and our research fields.

Researchers in the CDBRB have made substantial progress and exciting scientific breakthroughs during the past year as reflected in the 54 publications in our annual report bibliography. Several selected research advances are highlighted below. The project reports from each Section provide more comprehensive summaries of the major new findings in our Branch.

CDBRB initiated the Oral and Craniofacial Genome Anatomy Project (OC-GAP) to catalogue genes expressed in oral and craniofacial tissues and to discover novel genes important for tooth, oral, and craniofacial development. Previously, CDBRB researchers discovered ameloblastin, a tooth-specific, developmentally regulated gene associated with enamel formation and linked to the congenital disorder amelogenesis imperfecta. Members of our Branch have also discovered the cytoskeletal gene vinexin, which helps to regulate cell adhesion and the Sos-JNK signaling pathway. Other new genes currently being characterized may have important roles in developmental or disease processes. Hundreds of novel genes have been identified from cDNA libraries, and many genes show distinctive mRNA expression patterns in developing tissues. Recently, we partially sequenced an additional 1,447 clones randomly selected from a mouse embryonic 8.5-day craniofacial subtraction cDNA library and found that 32% of the clones were novel, with low sequence homology in GenBank database searches. Unique clones with interesting expression patterns are being characterized further. In addition, gene expression profiling of five different stages of salivary development has identified a variety of additional genes that are potentially important in gland development. CDBRB members also serve as Project Officers of a major new contract with Washington University, St. Louis, in association with research teams at Johns Hopkins University and the Necker-Enfant Malades Hospital in Paris to discover and catalogue expression patterns of human craniofacial genes that are active during early development. All clones, cDNA libraries, and antibodies will continue to be freely available to dental, craniofacial, and other investigators to promote research in the area.

Our knowledge about gene regulation and the extracellular matrix is being used to examine pathology in animal models and in human diseases. For example, the Molecular Biology Section has created gene knockout mice to study the biological roles of link protein and perlecan. Link protein-null mutants showed progressive dwarfism, suggesting a critical role for link protein in the maintenance of cartilage structure and function during skeletal development. Perlecan-deficient mice also showed severe chondrodysplasia, with radiographic and clinical features remarkably similar to a lethal autosomal recessive disorder in humans termed dyssegmental dysplasia, Silverman-Handmaker type (DDSH). We identified a homozygous 89-bp duplication in exon 34 of the perlecan gene (*HSPG2*) in a pair of siblings with DDSH born to consanguineous parents. The duplication mutation causes a frameshift, resulting in a truncated protein core that is not secreted by the patient's chondrocytes but is degraded to smaller fragments within cells. Thus, DDSH is caused by a functional null mutation of perlecan.

Laminin and laminin peptides have been implicated by CDBRB members in differentiation, tumor growth, and metastasis. Active sites of laminin chains have been identified in a variety of biological processes using synthetic peptide and recombinant protein approaches by the Molecular and Cell Biology Sections. Many of these peptides and molecules have potent cell type-specific effects on cell adhesion, angiogenesis, salivary gland differentiation, and tumor metastasis. For example, one peptide can cause tumor cells to metastasize to the liver, and another increases lung colonization by 5-fold. The receptors for both have been identified: the former is a cell surface heparan sulfate-containing molecule, and the latter is an integrin. These studies should lead to the development of new therapeutic reagents.

Little is known about how the extracellular matrix is organized by cells, e.g. to form patterns of fibrils in connective tissue. The Developmental Mechanisms Section has discovered a novel mechanism used by cells to create fibrils of fibronectin involving a linear movement of integrins along the cell surface. This movement appears to pull fibronectin into long fibrils, which eventually organize into a three-dimensional extracellular matrix. This process depends on the cytoskeletal protein tensin and cellular actomyosin contractility, but not cell migration. Integrin-mediated interactions of cells with such matrices induce signal transduction, cytoskeletal organization, migration, growth, and protection from apoptosis. MAP kinases have been implicated in these processes, and two novel signaling partners whose locations are regulated by integrins were identified. Tensin was found to be able to trigger JNK and p38 activation, and membrane-linked paxillin can trigger JNK activity. The capability of cytoskeletal proteins to trigger signal transduction suggests that adhesion complexes containing clusters of these proteins can be potential sources of signaling. Understanding the mechanisms of matrix assembly and signaling should eventually benefit cell-based tissue engineering.

The Cell Biology Section has identified potent extracellular regulators of cell migration and tumor metastasis. Ongoing studies on thymosin beta4 have established roles in endothelial, keratinocyte, and corneal epithelial cell migration. It decreases inflammation and accelerates wound repair in a rat skin model and in the cornea, suggesting its potential for promoting human wound healing. A novel function for osteonectin was identified as supporting breast and prostate cancer cell metastasis to bone and induction of proteases. The cellular receptor has been identified as an integrin, and the amount of this integrin may serve as a diagnostic marker for metastatic breast and prostate cancer.

The molecular responses to extracellular matrix are being characterized in human salivary gland (HSG) cells and fibroblasts by the Cell Biology and Developmental Mechanisms Sections. When cells are placed on extracellular matrix proteins in cell culture, they show large changes in gene expression and protein biosynthesis. More than two dozen genes were identified as induced by adhesion of salivary cells to collagen, fibronectin, or basement membrane extract, and many of them are novel. Expression of the metallothionein gene was found to be induced six-fold by laminin. When expression in salivary gland tumor cells is increased by transfection, the acinar structures formed in vitro are larger, and the tumors formed in vivo are smaller and more differentiated. This gene is likely to have a role in salivary differentiation. These studies are building the knowledge base necessary to develop creative therapeutic approaches for the repair or replacement of salivary glands and other tissues. For example, CDBRB is collaborating with the Gene Therapy and Therapeutics Branch to develop a first-generation artificial salivary gland.

Besides publishing extensively, our Branch distributes its research materials widely by licensing, donating to repositories, and providing numerous gifts to research colleagues. Products generated by CDBRB members that were licensed by companies included Matrigel, invasion substrates, and monoclonal antibodies against integrins. CDBRB has donated hundreds of cDNA clones to the ATCC and completed nearly a hundred new Material Transfer Agreements with extramural researchers this year to provide our reagents. Members of the branch have also received support from outside organizations. Significant support for research on proteoglycans came from Seikagaku. NASA provided funds to study salivary gland cell differentiation in microgravity. Non-NIH salary support for postdoctoral members of CDBRB has come from various sources.

CDBRB members continue to be invited as featured speakers at a variety of international meetings and symposia. Examples from this past fiscal year included the International Symposium on Craniofacial Development in Seoul, Korea (Y. Yamada), VIII International Congress of the Metastasis Research Society in London (H. Kleinman), the Keystone Symposium on Signaling Pathways by Integrins and Growth Factors (K. Yamada), the VI International Congress on Immunorehabilitation in Israel (H. Kleinman), as well as Gordon Conferences on Signal Transduction by Engineered Extracellular Matrices (K. Yamada), Proteoglycans (Y. Yamada), Biomineralization (H. Kleinman), and Signaling by Adhesion Receptors (K. Yamada). Our members continue to serve on the editorial boards of a number of leading journals. Examples include J. Cell Biology (K. Yamada, Editor, and H. Kleinman, board member); Cancer Research (H. Kleinman, Associate Editor); J. Cellular Physiology (K. Yamada, Editor); Matrix Biology (Y. Yamada and K. Yamada, Associate Editors); J. National Cancer Institute (H. Kleinman, Associate Editor); J. Biological Chemistry (Y. Yamada, board member), and J. Cell Science (K. Yamada, board member). H. Kleinman also serves on 4 other journal boards and K. Yamada serves on 5 others. Members also served on U.S. Army study sections and on the Board of the Metastasis Research Society (H. Kleinman), and on the Council of the International Society for Matrix Biology (K. Yamada). CDBRB members provide extensive service on more than two dozen NIH and NIDCR committees, including the NIH Senior Biomedical Research Service Policy Board, NIH Diversity Council, NIH Scientific Conduct and Ethics Committee, and NIDCR Tenure and Promotion Committee.

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Craniofacial Epidemiology and Genetics Branch

Scott Diehl

CRANIOFACIAL EPIDEMIOLOGY AND GENETICS BRANCH
2000

The mission of the Craniofacial Epidemiology and Genetics Branch (CEGB) is focused on using epidemiological strategies and approaches to enhance understanding of the hereditary and environmental causes of dental, oral and craniofacial diseases and disorders. Such knowledge can be used to improve diagnosis, prevention and therapy. Most of the research conducted in the Branch involves clinical research conducted on human subjects. Limited studies are conducted using animal models. Basic research involves the development and evaluation of new laboratory (genomic) assays and statistical methods designed to improve the power and efficiency of these research approaches, followed by application to ongoing studies being conducted in the Branch and elsewhere. Current studies of CEGB staff include large surveys of oral and systemic health based on interviews and examinations, case-control and cohort designs, and recruitment of nuclear and extended families. The focus of these investigations range from assessing health effects of amalgam, characterizing the oral manifestations of HIV and the oral physiology of aging, and improving understanding of the etiology of periodontitis and other causes of tooth loss, oral cancer, nasopharyngeal carcinoma, cleft lip and palate and other craniofacial disorders. Most studies are designed to evaluate both genetic variation and the behavioral and environmental risk factors associated with these diseases. Three Senior Investigators have conducted research in the CEGB of the Division of Intramural Research (DIR), NIDCR during the past year: Scott R. Diehl, Ph.D., Deborah M. Winn, Ph.D., and Albert Kingman, Ph.D. Most of the CEGB staff work at offices and computer facilities located in the Natcher Building on the main NIH campus. In addition, Dr. Diehl's gene mapping laboratory is located a short distance away at the National Naval Dental Center.

Molecular Genetic Epidemiology

The mission of Dr. Diehl's research program is to increase our understanding of the etiology of dental, oral and craniofacial disorders by using the gene mapping strategies of association (disequilibrium) and linkage. Nearly all of his studies involve complex diseases, where multiple susceptibility genes are involved, and where gene-environment interactions are common. Although the majority of his studies utilize molecular assays such as marker polymorphisms or mutation analyses of candidate genes, several projects investigate familial aggregation of diseases and disease-related phenotypes without incorporation of molecular data. Analyses include key behavioral risk factors such as diet, smoking and alcohol consumption. These are treated both as covariates to disease risk and as genetically heritable phenotypes of interest themselves. The gene mapping strategy requires the following components, all of which have been successfully implemented by Dr. Diehl's research team: design and implementation of large scale field studies to obtain biospecimens and risk factor assessments for cases and appropriately matched controls; establishment of a laboratory capable of high-throughput genomic assays for both highly polymorphic DNA markers and candidate gene single nucleotide polymorphisms (SNPs); bioinformatics systems for management of biospecimens, molecular assay data, and clinical and risk factor information; and construction of computer hardware and software systems capable of conducting the thousands of statistical analyses required for genome wide studies by

integrating a diverse array of computer programs in a user-friendly, semi-automated environment.

Dr. Diehl has collaborations with clinicians and epidemiologists throughout the world that have led to the successful establishment of several large patient collections for his research. Oral cancer cases and controls have been recruited in Europe, North American and Asia, using both case-control and family-based sampling designs. A gene mapping and risk factor study of nasopharyngeal carcinoma has been implemented in Taiwan, based primarily on multiplex families (families with 2 or more affected members). A large study of syndromic and non-syndromic cleft lip and palate using both simplex and multiplex families has been completed at three clinical sites in the U.S. Studies of oral clefts in humans have been complemented by Quantitative Trait Locus (QTL) analyses of a mouse model of teratogen-induced clefting. New studies of pain using human subjects suffering from phantom limb pain and the autotomy animal model have been initiated in collaboration with scientists at the NIDCR and from Israel. A study of Kartagener syndrome has led to the mapping of a disease gene using families obtained through a collaboration in Poland. Kartagener syndrome is a form of primarily ciliary dyskinesia that has chronic sinusitis as a craniofacial manifestation. A whole genome scan and analyses of several candidate genes is being conducted for a study of early onset periodontitis using families collected in the U.S., Chile, and Israel.

Completion of these major, long-term studies, several of which are currently in subject recruitment phases, will be the focus of much of Dr. Diehl's research team's efforts during the next couple of years. Improvements in genomic technologies, especially the exciting developments in the area of single nucleotide polymorphisms (SNPs) can be expected to vastly increase the speed and scope of analyses that will be feasible for gene mapping laboratories. Dr. Diehl is committed to keeping his laboratory at the cutting edge of these new methods. Considering these likely technological advances in laboratory capabilities, ensuring future access to large numbers of biospecimens with high quality risk factor and demographic data will become an even more essential priority if this research program is to remain highly productive. Long term planning is especially important in this field, since it can take many years to establish collaborations, design and carry out large epidemiological studies.

Analytical Epidemiology

The staff of the Analytical Epidemiology component, under the direction of Dr. Deborah Winn, has continued a program of scientific accomplishment and professional service. Their Intramural research includes studies of periodontal health and of oral and pharyngeal cancer. Early onset periodontitis (EOP) is a disease characterized by a progressive loss of the tooth supporting tissue in adolescents and young adults. In one study, a group of adolescents with early onset periodontitis and a control group of adolescents received an initial oral examination and a follow-up examination six years later. Advances have been made in understanding how levels of serum antibodies to P. gingivalis, P. intermedia, and A. actinomycetemcomitans relate to levels of these antibodies in gingival crevicular fluid, and how levels differ in control subjects and in generalized and localized forms of EOP. Based on analysis of data from another study population, the Baltimore Longitudinal Study of Aging, pipe and cigar smoking appear to have the same adverse effects on periodontal disease as cigarette smoking. This finding is important

in view of the recent surge in the popularity of cigar smoking. In another study, our analyses suggest that infection with P. gingivalis has a different pattern than for A. actinomycetemcomitans in the U.S. population, which may have implications for periodontal disease patterns in demographic and risk factor subgroups of the population.

Oral and pharyngeal cancers are characterized by a) alcohol- and tobacco-related etiology, b) low proportion of tumors identified at an early stage, c) racial differences in stage at presentation, d) racial disparities in survival even after controlling for stage, and e) approximately 50% survival rate. Several ongoing interrelated studies have as their goal the identification of risk factors for oral and pharyngeal cancer and identification of factors that influence detection of oral cancer or of persons at highest risk of these cancers. One project is the Puerto Rico Oral and Pharyngeal Cancer Study in which patients identified through a cancer registry are compared to the general population. The purpose is to identify genetic, behavioral, medical, and familial factors involved in the etiology of this disease. Recent key findings include evidence that oral and pharyngeal cancer risks decrease with cessation of tobacco and alcohol consumption, although risks of these cancers remains elevated for up to 20 years after cessation of use of these substances.

The SEER/Medicare Linkage Project is focused currently on a determination of patient medical care contacts occurring in the year prior to diagnosis of oral and pharyngeal cancer and the reasons for those contacts. Work to date suggests that physician visits are associated with earlier stage diagnosis for pharyngeal and laryngeal cancers, but not oral cancers. More seriously ill users of physician services are at greater risk for late-stage at diagnosis than those not as ill. These findings have implications for development of interventions for early detection of these cancers. In a related analysis, we have recently discovered a striking increase over time in the incidence of in-situ carcinomas of the oral cavity, pharynx and larynx.

Dr. Winn recently moved to the NCI where she will continue many of these research studies that are focused on oral cancer in collaboration with NIDCR scientists including Dr. Diehl.

NIDCR Chief Statistician

Dr. Albert Kingman has continued to serve in his capacity as NIDCR's Chief Statistician. His primary research interests include the following three areas: 1) design and analytical issues related to randomized clinical trials, including design issues in equivalence trials, with appropriate and efficient use of surrogate and multiple endpoints; 2) development of methodology in statistical genetics, focusing on linkage analysis and association studies; and 3) statistical issues in environmental risk assessment, especially related to exposure to dental amalgam and its potential health effects. Dr. Kingman is currently pursuing part-time sabbatical training in the field of genetic epidemiology at the Center for Inherited Disease Research in Baltimore. This center is a gene mapping facility jointly managed by the NIH and Johns Hopkins University. As part of this training experience, he is currently collaborating on several genetic epidemiology studies and working on new approaches to linkage analysis and association studies for human genome screens involving thousands of markers. He currently heads the Biostatistics Core which provides training and consultation to the entire NIDCR on statistical design and analysis issues.

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Craniofacial and Skeletal Diseases Branch

Pamela Gehron Robey
Larry W. Fisher
Marian F. Young

CRANIOFACIAL AND SKELETAL DISEASES BRANCH
2000

This Branch has focused its efforts on determining the processes by which skeletal elements are modeled during embryogenesis, remodeled and maintained in the post-natal organism, and how these processes are altered in disease states through the coordinated efforts of senior investigators working in the areas of developmental biology, cell and molecular biology, and protein and mineral chemistry. Furthermore, the translation of these efforts into clinical studies and applications has been, and remains a major area of emphasis in the Branch. The Branch has active collaborations with other NIH Institutes and institutions around the world, and participates in the Georgetown University Orthopaedic Residents Training Program. The Branch has made major advances in several areas, as highlighted below.

Studies in the Developmental Biology project performed by Dr. J. Terrig Thomas are aimed at understanding the molecular basis of limb development and joint morphogenesis with special emphasis on signaling molecules. Frzb is a secreted protein, initially isolated from purified cartilage extracts, which shares homology to the cysteine rich domain (CRD) of the frizzled family of Wnt receptors. The Wnt proteins are secreted signaling molecules having numerous developmental functions, including skeletal development, as well as dysfunction in oncogenesis. It has been shown that Frzb can bind to and inactivate Wnt activity, leading to speculation for its potential therapeutic use in modifying Wnt induced developmental and oncogenic events. We have demonstrated that Frzb is temporally and spatially expressed during skeletal and craniofacial development. In an attempt to understand the role of Frzb in development we conducted a conditional gene knockout, using the Cre-LoxP recombination system. We generated "floxed" mice, in which LoxP sites flank the Frzb gene, by homologous recombination. These mice were subsequently crossed to a transgenic mouse line expressing Cre recombinase under the control of a ubiquitous promoter to delete Frzb early in development. The null phenotype, however, appears normal. This is most likely due to functional redundancy, as there are now known to be a number of related Frzb-like genes with overlapping expression patterns. Consequently, in order to study the role of these genes during development it will be necessary to carry out double knockouts of different family members. It should still be possible to determine the role of Frzb in limb and face development by crossing the floxed Frzb mice to null mice for other family members once they are available. Subsequently, Frzb can be deleted in a tissue-specific manner by crossing the mice with transgenic mice expressing Cre recombinase under the control of tissue-specific promoters. In order to do this we are generating transgenic mouse lines to express Cre recombinase specifically in the limb and facial mesenchyme. These mice are currently being tested.

The Skeletal Biology unit, directed by Dr. Pamela Gehron Robey, has focused on bone marrow stromal cells (BMSCs), which have the ability to form bone, cartilage, hematopoiesis-supportive stroma, associated fat cells, and perhaps other connective tissues as well, and are important mediators of skeletal metabolism in the post-natal organism. Previously, we had found that some, but not all, of the members of the stromal cell population maintain their ability to form bone, hematopoiesis supportive stroma and associated adipocytes. The differences between bone forming and non-bone forming clones were further investigated by examining their expression of

transcription factors, cytokines and matrix proteins. c-Myc and c-Jun did not differ between osteogenic and non-osteogenic clonal strains, whereas c-Fos expression was higher in osteogenic clones. IL-6 and IL-11 expression was significantly higher in osteogenic clones than in non-osteogenic clones. Differences in ECM expression were noted only with respect to the level of biglycan, which was higher in osteogenic versus non-osteogenic clones. Similar studies have been performed on five spontaneously transformed murine stromal cell lines by looking at the expression profiles of phenotype-characteristic genes, patterns of in vitro differentiation and osteogenic capacity after in vivo transplantation. All the clones expressed stable levels of Cbfa1, the osteogenic "master" gene, collagen type I and AP expression were common to all, and therefore presumably early, basic traits of stromal cell lines that otherwise significantly differ with respect to growth and differentiation potential as measured by patterns of gene expression. This finding suggests that an osteogenic imprinting lies upstream of diversification, modulation and restriction of stromal cell differentiation potential. Our results extend current knowledge on the differential expression of various genes by hBMSCs, correlated with their osteogenic potential. In collaboration with investigators at the University of North Carolina, we have used a new approach to isolate and characterize human cementoblasts for the first time. These cells were found to be similar, but not identical to cells that produce bone. This provides an excellent model system to study the physiology of this unusual cell type, and for future design of periodontal reconstructions. The Unit has continued its collaboration with members of NHGRI in the Skeletal Genome Anatomy Project (SGAP) which is designed to aid in gene discovery and to determine changes in the pattern of gene expression of skeletally derived cells as a function of developmental age and of disease processes. To date, over 10,000 clones from two of our libraries have been sequenced and approximately 30 are novel. We have characterized one such novel clone that codes for a protein with a highly unusual combination of zinc fingers that we have named Double FYVE-containing protein 1 (DFCP1). The gene, *ZNFN2A1* (GenBank entry AF251025) was localized to chromosome 14q22-q24. DFCP1 appears to be expressed in a variety of different tissues, especially in endocrine tissues. Following in vitro transfection of a DFCP1-containing expression construct, confocal microscopy studies showed a vesicular distribution of DFCP1 suggesting that this protein, like other FYVE-containing proteins, might be involved in membrane trafficking.

The matrix proteins of bones and teeth play key roles in the structure and functions of these tissues. The objective of the Molecular Biology of Bones and Teeth unit, lead by Dr. Marian F. Young, was to study their function and regulation using a combination of in vitro and in vivo analysis. To determine the function of matrix proteins in vivo, we generated transgenic animals that are deficient in one or more Small Leucine Rich Proteoglycans (SLRPs). SLRPs are an expanding family of proteoglycans found in the extracellular matrix that contains multiple tandem repeats of a motif rich in leucine. The most abundant SLRP in bone is biglycan (BGN) and is, currently, the major focus of our studies. To determine the functions of BGN in vivo, transgenic mice were created that were deficient in the production of the protein (knockout/KO). These mice acquired diminished bone mass that was progressive with age. Double tetracyline-calcein labeling revealed that the BGN deficient mice were defective in their capacity to form bone. To determine the cellular basis for the skeletal defect, bone cells were isolated from the marrow of normal and mutant mice at 6, 12 and 24 weeks of age and the number of bone cell precursors (CFU-f) quantitated. A dramatic age dependent decrease in colony forming units was observed in the BGN KO animals compared to normal littermates. Northern analysis showed

that type I collagen mRNA was also diminished indicating that both the quantity and quality of the bone forming cells was affected in the BGN KO mice. Electron Microscopy (EM) analysis showed that bones isolated from BGN KO mice had collagen fibrils that were more "irregular" in shape. Such structural defects may be causative factors in the compromised bone mineralization and biomechanical strength previously observed in the mutant mouse lines. Western analysis of the bone showed that BGN KO mice contain substantial amounts of the highly related SLRP decorin (DCN). To determine if DCN additionally contributes to bone tissue integrity we generated mice deficient in both BGN and DCN. The "double KO" mice had substantial decreases in both trabecular and cortical bone mass compared to WT, BGN or DCN KO animals implicating a compensatory role of DCN in the absence of BGN. A major challenge will be to understand how gene expression patterns are altered in the SLRP KO mice and, further, how the responses of growth factor and hormones are affected. These studies will be necessary to untangle the complex network of SLRP function in bone at the tissue, cell and molecular levels

The fundamental question of how cells of bones and teeth assemble and mineralize their respective matrices in such a coordinated and superbly biofunctional way is still largely unanswered. The Matrix Biochemistry Unit, headed by Dr. Larry W. Fisher, has been performing a variety of experiments and collaborations to help determine the structure-function relationship of several of the more interesting noncollagenous proteins. The SIBLING family of integrin-binding matrix skeletal matrix proteins that include bone sialoprotein (BSP), osteopontin (OPN), dentin matrix protein 1 (DMP1) and dentin sialophosphoprotein, which are closely related proteins, were recently found to be in close relationship to one another on 4q21-23. With our colleagues, we have extended our previous observations of BSP expression in primary breast cancer by development of an assay that detects BSP in the sera of patients with a number of different cancers. We have discovered that at least BSP, OPN and DMP1 can bridge complement Factor H to integrins or CD44 (for OPN and DMP1) and inhibit the lytic pathway of complement, leading to the hypothesis that BSP expression by primary tumors may allow tumor cells to escape immune surveillance. Furthermore, BSP has been shown to mediate human endothelial cell attachment and migration, and to promote angiogenesis, a process that is of extreme importance not only in bone formation, but in tumor growth. In addition, the unit has worked with other members of the branch in developing a better understanding of McCune-Albright Syndrome (MAS) and Fibrous Dysplasia. We have continued to develop a new assay that aids in the rapid detection of the mutation and is able to detect 1 mutant cell out of 100. By using this technique, it is now apparent that the nature and severity of a lesion is highly dependent on the degree of somatic mosaicism.

Dr. E. D. Eanes, who retired on October 1, 2000, headed the research of the Mineral Chemistry and Structure Section. Mr. Arthur Hailer has been reassigned to the Office of the Chief, and is currently training in website and database development. Mr. Bruce Fowler, who will be retiring on September 30, 2000, continued to focus on biologically relevant calcium phosphate salts. The main objective was to determine compositional and structural details of the inorganic phase in teeth and bones. Infrared and Raman spectroscopy, x-ray diffraction and chemical methods are employed in these studies. A better understanding of the compositional and structural details of the apatite mineral phase in teeth and bones has been made possible by studies on its synthetic analogs. Consequently, methods are devised for the preparation of synthetic calcium apatites having controlled physical properties (crystal size and shape and crystal perfection) and chemical

constituents (hydroxide, fluoride, chloride, carbonate, water, acid phosphate and other ions). Isotopically enriched apatite analogs are also prepared to facilitate spectral assignments. The vibrational spectra of these apatites and related compounds are assigned using factor group symmetry methods. The spectroscopic band assignments and supplemental data (temperature dependence and polarization of bands) are then utilized to establish compositional and structural details of the apatites in question, which include: the type and geometry of constituent ions; the site and number of sites occupied by the ions; orientation of ions; chemical bonding and interactions of ions; and semi-quantitative estimations of constituents present. The results of these controlled apatite systems are then related to the inorganic phase in calcified tissues. The infrared and Raman spectroscopic and x-ray diffraction methods are also utilized in dental materials research to determine and monitor reactions during syntheses of various inorganic and organic compounds used for composites, coupling agents, cements and other dental materials. A new calcium bisphosphonate, calcium glutaryl-bisphosphonate was prepared and characterized. X-ray diffraction results show an epitaxial relationship with apatitic surfaces, which suggests that this compound may have clinical use in treating calcium-related bone disorders.

The Skeletal Clinical Studies program, currently under the direction of Dr. Pamela Gehron Robey, has established four clinical protocols (97-DK-0055, 98-D-0145, 98-D-0146, 99-D-003) for the study and treatment of fibrous dysplasia of bone (FD) and the McCune-Albright Syndrome (MAS). A fifth protocol, "Effects of the Aromatase Inhibitor Letrozole on Pubertal Progression and Indices of Bone Turnover in Girls with Precocious Puberty and McCune-Albright Syndrome (MAS)", funded by a Bench to Bedside award to NIDCR and NICHD, has recently been approved (00-D-0183), and will begin patient accrual shortly. FD is found in a broad range of severities, ranging from monostotic (single bone) to polyostotic (many bones) and often in association with the MAS, which in addition to FD has multiple endocrinopathies and skin hyperpigmentation. MAS is known to arise from a post-zygotic mutation in the GNAS1 gene (R201C and R201H). In an in-depth histological and molecular study of FD lesions, aided by the development of a novel assay by Dr. Larry Fisher, all of the patients were found to have GNAS1 mutated cells. In addition, we also identified a novel R201G mutation in one of our patients. These results indicate that GNAS1 mutations result in a broad spectrum of bone lesions even outside of the context of MAS, and that new therapies may have a broader impact than previously realized. In addition to our studies of disease, we are developing new techniques for bone repair. Although it has been known since the 1960's that bone marrow stroma contains a population of cells that have the ability to form bone, cartilage, myleosupportive stroma, adipocytes and perhaps other connective tissues, it is only recently that their utilization for bone regeneration has been fully realized. The Unit is developing procedures to optimize ex vivo expansion of bone marrow stromal cells, and transplantation for tissue regeneration. It was determined that if fetal bovine serum is removed from the cells prior to transplantation, as would be required for use in human patients, bone formation was actually increased. Furthermore, it was determined that the size of hydroxyapatite/tricalcium phosphate particles use as a carrier also influences the amount of bone formation. In addition, it was also determined that the ex vivo expanded cells could be used to generate vascularized bone grafts, a technique that has major utilizations in reconstructive surgery. These studies have served as the basis for development of procedures for use in humans non-healing bone defects. An application has been submitted to the FDA, and it is hoped that clinical trials will begin in the very near future.

CRANIOFACIAL AND SKELETAL DISEASES BRANCH
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Gene Therapy and Therapeutics Branch

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GENE THERAPY AND THERAPEUTICS BRANCH
2000

The Gene Therapy and Therapeutics Branch (GTTB) is a model of translational research, providing a bench to clinic continuum. The GTTB has both a tissue-specific focus, asking questions related to salivary gland biology and pathology, as well as an applications focus, gene transfer technology. The GTTB remains committed to the notion that significant advances in clinical care will come from our understanding of biological mechanisms and our inter-disciplinary approach. This reporting period has seen not only substantial scientific progress, but also significant changes in our physical facilities. GTTB laboratories underwent substantial renovations. These provided us with a modern biomedical research environment, and have been a wonderful boost to staff morale.

The production of salivary fluid is due to the transepithelial secretion of Cl^- by acinar cells. A Na^+-K^+-$2Cl^-$ cotransporter, NKCC1, located in the acinar basolateral membrane drives much of this Cl^- flux. This transporter is the rate-limiting step in salivary secretion. The Membrane Biology Section (MBS) continues to concentrate on understanding the structure, function and regulation of this important transport protein. They have recently developed a permeabilized rat parotid acinar cell system to study NKCC1 regulatory events *in situ*. In this system they can mimic the upregulatory phosphorylation of NKCC1 induced by β-adrenergic stimulation and this has allowed them to tentatively identify the phosphoamino acid involved. To gain more information about the structure of NKCC1, the MBS used different theoretical algorithms to predict its transmembrane topology. Several similar, but not identical, structures were predicted. MBS investigators then experimentally explored the actual structure by testing the ability of putative membrane spanning segments (MSSs) to integrate into microsomes *in vitro*. By using expression vectors that appended a glycosidation tag to the C-terminus of the MSSs being tested, they were able to assay for both signal anchor and stop transfer activities. Combining this information with earlier topological data from their group, these studies clearly indicated a topology scheme consisting of 12 MSSs, including two pairs of tight hairpin-like structures within the membrane. Interestingly, none of the theoretical methods correctly predicted the locations and orientations of these two hairpin-like structures. More detailed structural studies require a large source of functional, pure NKCC1 protein. To achieve this MBS staff have transformed the yeast, *S. cerevisiae*, with the NKCC1 cDNA and shown that the yeast can make >10 fold higher levels of NKCC1 than rat parotid gland, one of the richest sources known for this protein.

Neurotransmitter stimulation of sustained salivary fluid secretion depends on the activation of store-operated Ca^{2+} entry (SOCE). The molecular mechanism involved in this process is unknown for all non-excitable cell types. GTTB's Secretory Physiology Section (SPS) is focused on identifying the component(s) mediating Ca^{2+} influx using salivary gland cells as models. SPS scientists made exceptional progress during this reporting period on their studies of the transient receptor potential *(trp)* gene family as possibly encoding the store-operated Ca^{2+} influx channel (SOCC). For example, they demonstrated the single channel properties for the SOCC in the HSG cell line. This human submandibular line has proven an extremely valuable model for studying SOCE. The SPS studies showed the SOCC to have unitary slope conductances of 19-

20 pS, and to be inhibited by lanthanum and gadolinium, but not zinc. Notably, the Trp1 channel has been reported to have a similar conductance for Ca^{2+}. These studies also show that the kinetic properties of the SOCC are determined by the function of the inositol trisphosphate receptor and the intracellular Ca^{2+} pump. To assess Trp protein function SPS investigators constructed an adenoviral vector encoding human (h) Trp1, AdHA-hTrp1, and used this vector to infect both HSG cells *in vitro* and rat submandibular glands (SMGs) *in vivo*. The vector led to a dose-dependent expression of hTrp1 in HSG cells and SMGs, as well as increased SOCC activity. In the SMG hTrp1 was located in the basolateral membrane and glands infected with AdHA-hTrp1 exhibited increased salivary fluid secretion after stimulation with pilocarpine. To help understand the mechanism by which hTrp1 functions, the SPS constructed a truncated form (deletion of amino acids 664-793; the C-terminus). The expression of this truncated form was without effect on basal SOCC activity. However, after store-depletion (by carbachol or thapsigargin) SOCC activity associated with the truncated Trp was much greater than that of wild type. The truncated form, like the wild type, was localized in a lipid raft microdomain, and was immunologically co-precipitated with caveolin-1 and the inositol trisphosphate receptor. In aggregate, these latter studies show that the C-terminus of hTrp1 is not involved in the gating or localization the SOCC, but most likely plays a modulatory role. Thus, these studies suggest that SOCE is localized in specialized microdomains and that the SOCC is assembled in a complex with other proteins involved in its regulation.

The newly established AAV Biology Unit (AAVBU) has made remarkable progress during its first year. The focus of this group's activity is on understanding the interactions of adeno-associated virus (AAV) with its host cell. As a helper-dependent parvovirus, AAV requires a considerable degree of host cell support for transduction and life cycle events, much of which is poorly understood. The AAVBU staff has cloned two novel AAV serotypes (AAV4 and AAV5) and shown *in vitro* and *in vivo* that these vectors may be useful to mediate gene transfer to cells and tissues poorly transduced by the commonly utilized serotype 2 AAV vectors. For example, a recombinant serotype 5 vector is ~50 fold more efficient in mediating gene transfer to cultured human airway epithelia than an AAV2 vector. This observation potentially may have significant clinical impact. Other AAVBU studies have shown specific interactions between AAV Rep (viral regulatory) proteins and important cellular cyclic AMP response factors (protein kinase A; CRE binding protein) in target cells, which can lead to a broad array of effects on host cell processes.

The Gene Transfer Section (GTS) studies clinically relevant applications of gene transfer to salivary glands. Historically, much of this work has employed replication-deficient recombinant adenoviral vectors. While these vectors are useful for establishing proofs of clinical or biological principle, adenoviral-mediated transgene expression is transient and vectors elicit a potent immune response. Accordingly, the GTS greatly increased its efforts to develop alternative means of transferring genes to salivary glands. For example, during this reporting period the GTS extended their earlier work with recombinant AAV serotype 2 (rAAV2) vectors. We constructed a rAAV2 encoding human interleukin (hIL)-10, rAAVhIL10, and showed that it directed the production of biologically active hIL-10. In murine submandibular glands hIL-10 expression was stable for >2 months, the longest time studied, and virtually all of the IL-10 produced was secreted into the bloodstream, i.e. in an endocrine direction. They have also continued their efforts towards practical application of the hybrid adeno-retroviral vector

described last year. The prototype hybrid vector carries the 5' and 3' long terminal repeat (LTR) sequences from Moloney murine leukemia virus (MoMLV), and a luciferase reporter gene in an adenoviral type 5 backbone. They showed that this vector can infect dividing and non- dividing cells *in vitro*, and submandibular glands and brain *in vivo*, as well as integrate into the host cell genome. The latter is atypical for MoMLV integration and occurs in the absence of trans-complementing integrase. This year the GTS focused on understanding the mechanism by which integration occurs, constructing a series of vectors with deletions in either the 5' or 3' LTRs in order to define required elements. Interestingly, they observed that a hybrid vector containing only the 5' LTR is still able to achieve integration.

The GTS previously showed that salivary glands are able to secrete transgene-encoded proteins into serum as well as saliva, and that transgene-encoded therapeutic proteins can follow distinct sorting pathways *in vivo*. Much of the effort in this area has focused on the sorting signals involved, and how they can be manipulated. The GTS used growth hormone (GH) as a model, transgene-encoded secretory protein in our studies. Last year they showed that GH was primarily secreted via the regulated pathway into saliva. The GTS goal is to re-direct the GH secretion into the bloodstream where it is clinically relevant. They hypothesized that alkalinization of intracellular vesicles carrying GH in the regulated pathway would lead to their mis-sorting and their secretion via a constitutive pathway into the circulation. To achieve the alkalinization they used Plaquenil, at doses/kg normally used to treat patients with autoimmune disorders. This maneuver led to a dose-dependent re-direction of GH into the bloodstream, with little effect on GH synthesis or overall secretion. It is anticipated that this finding will prove to be clinically applicable.

Salivary epithelial cells can selectively sort proteins to apical or basolateral membranes. This asymmetric distribution is essential for the vectorial transport of water, electrolytes, and proteins in acinar cells during saliva formation. The GTS wishes to understand the sorting of salivary membrane proteins to facilitate the design and application of bioengineering strategies for salivary gland disorders, e.g. their ongoing efforts to develop an artificial salivary gland. During this reporting period they used confocal microscopy, and primary antibodies directed at aquaporin (AQP) 8, to localize endogeneous AQP8 in the basolateral membranes of rat SMGs, i.e. distinct from the apical localization which we previously established for AQP5. They next tried to develop an *in vitro* model system to study AQP sorting, using MDCK-II cells stably expressing either AQP5 or AQP8 under the control of a tetracycline-regulatable CMV promoter. Both expressed aquaporins were functional and mediated osmotically obliged fluid movement. Importantly, AQP5 sorted to the apical membranes, while AQP8 sorted to the basolateral membranes, in MDCK-II cells. Thus, the GTS has established a useful system with which to determine the sorting signals used by AQPs 5 and 8 to achieve their polarized distribution.

GTTB clinical studies focus on determining if the salivary dysfunction accompanying Sjogren's syndrome (SS) can be ameliorated by immunomodulatory therapy. Our SS Clinic just completed a trial of Plaquenil (vs pilocarpine as an active placebo), and showed that certain systemic manifestations of SS disease activity are significantly improved (e.g. serum IgG levels, erythrocyte sedimentation rate), as are salivary flow rates. Currently, three additional clinical trials for SS are in process and actively accruing patients. These include randomized, placebo controlled clinical trials of dehydroepiandrosterone, thalidomide, and etanercept, the latter two

both being tumor necrosis alpha antagonists. The SS Clinic is also actively involved in establishing international collaborations with multiple centers involved in rheumatic disease research to develop outcome measures that are appropriate for longitudinal studies and clinical trials in SS.

GENE THERAPY AND THERAPEUTICS BRANCH
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Oral Infection and Immunity Branch

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ORAL INFECTION AND IMMUNITY BRANCH
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The Oral Infection and Immunity Branch plans, fosters and carries out research relating to the causes, diagnosis, treatment and prevention of infectious and inflammatory diseases. Efforts to understand the functional and molecular organization of infectious organisms, and research into the cellular, biochemical and molecular components of inflammatory, immune and sensory responses provide the basis for dissecting the interactions between pathogens, noxious agents and the host defense system. These multifaceted approaches define fundamental mechanisms of host defense, how these pathways become dysregulated to become pathogenic, and how to intervene for the benefit of the host.

The Oral Infection and Immunity Branch (OIIB) continues to generate diverse and exciting advances in our research portfolio. The programs within the Branch range from basic through translational research, which fuels our fledgling clinical program. An important component of our commitment to the pursuit of clinical research is the joint training program with Children's National Medical Center for Infectious Disease Fellows and with the Department of Periodontology at the University of Maryland for Periodontal Disease Fellows. In addition to training and mentoring clinical and basic science fellows, Senior Investigators in the Branch continue to receive recognition for their successful research programs and service-oriented activities, including the sharing of discoveries and novel reagents with the extramural community. Our Senior Investigators received numerous invitations to organize, chair, and speak at international meetings, to participate on editorial boards, invitations to edit books and write authoritative chapters, and to serve as officers in scientific societies, all reflecting the stature of our staff in the scientific community. Within the Branch, OIIB Employee of the Quarter Awards also recognized special achievements.

The Branch has nine Senior Investigators who have made substantial progress in their unique research programs this past year through independent and collaborative endeavors. Important new findings and scientific breakthroughs, including those featured in prestigious journals such as *Cell*, *Proceedings of the National Academy of Science*, *Nature Medicine*, and *Journal of Biological Chemistry*, are highlighted in this Annual Report. Research in the Branch continues to focus on the involvement of specific bacterial components in microbial adhesion, colonization and the subsequent initiation of inflammation. Pivotal to the adhesion process in oral biofilms is the synthesis and expression of receptor polysaccharides on streptococci, and the genetic regulation of the biosynthesis of nucleotide-linked sugars as well as the formation, transport and polymerization of oligosaccharide repeating units into polysaccharide molecules is under close scrutiny. Multiple genes have been identified to play a role in this process and inactivation of selected key genes may lead to abolition of growth, adhesion or colonization. For the first time, green-fluorescent protein has been used to label and track *S. gordonii,* enabling identification of their binding targets and interactions. Among the commonly encountered binding partners in biofilms, *Fusobacterium nucleatum* co-aggregates not only with streptococci and other periopathogenic bacteria, but also with host cells through its cell-surface galactose-binding adhesin. Serving as a bridge between early and late colonizers, *Fusobacteria* have become a central focus in the Branch for dissecting out the genetic basis underlying their unusually broad

range of cellular and bacterial co-aggregations, which may then lead to design of methods to disrupt these adhesive pathways. Both *Fusobacteria* and *Streptococci* metabolize dietary amino acids and sugars to produce cytotoxic products including mercaptans, sulphides and organic acids which contribute to the etiology of tooth demineralization and oral inflammatory diseases. In identifying and elucidating the catalytic mechanisms comprising these pathways, a unique family of phospho-glycosylhydrolases has been identified. Following successful crystallization of these enzymes, collaborative efforts between investigators in OIIB and the University of York will solve the structure and catalytic mechanisms. Unexpectedly, *K. pneumoniae* possesses this same hydrolase and grows on sucrose, whereas *S. mutans* does not, implicating this enzyme as a pathogenic factor. Another genus of bacteria purportedly isolated by others from the oral cavity was described as an unusual group of small microorganisms capable of evoking pathological extraskeletal calcification. In definitive new observations, the existence of this controversial bacterial genus has been refuted by OIIB scientists and in fact, the biomineralization previously attributed to nanobacteria is likely initiated by nonliving macromolecules, and transferred on subculture by self-propagating microcrystalline apatite.

Innovative efforts continue in defining mechanisms whereby the toxin from *Bacillus anthracis* delivers its lethal blow to susceptible target cells, including X-ray crystallography, identification of the receptor, gene expression and signal transduction. Not only are these studies pivotal to the development of vaccines and toxin blockers, but perhaps more importantly, since *B. anthracis* has evolved an incredibly efficient targeting mechanism, to utilize the toxin as a cell-specific delivery vehicle. Although anthrax toxin, composed of 3 distinct proteins, is cytotoxic, its individual components are not, but rather have unique properties which can be exploited to construct therapeutic agents. For example, protective antigen protein (PA) binds to a cell surface receptor, is proteolytically cleaved by furin, complexes, and enters the cell. To achieve cell specific delivery of toxin fusion proteins, PA has been genetically altered to replace the furin cleavage site with a sequence recognized by tumor specific enzymes. These fusion proteins become highly toxic for tumor cells, providing the impetus for testing in animal tumor models. Additional fusion proteins with unique cell specificities and functions as immunogens in the induction of cellular immunity to viruses and other antigens are in development. Within the Branch, related efforts are directed at defining new approaches to therapeutic intervention and antibiotic resistance in bacterial and viral infections. Improved methods for generation of genetically detoxified pertussis toxin underlie enhanced acellular vaccine production against *B. pertussis*. In addition, transient expression of candidate antigens or protective antibodies in transgenic plants may facilitate vaccine development to bacterial and viral pathogens.

Infection and injury elicit a complex series of reactions in the host designed to isolate and/or eliminate the inciting agent(s), as well as minimize and repair tissue damage. Precise regulation of these mechanisms is crucial for the maintenance of tissue integrity, and malfunction may result in pathologic responses and/or tissue destruction. Mast cells contribute to such reactions by releasing an array of mediators following receptor-mediated intracellular signal transduction. Based on earlier data that FceR1-induced signaling was dependent on the protein tyrosine kinase Syk, Syk-negative mast cells were exploited by investigators in the Branch to further delineate the events which trigger mediator release. Newly identified links in the signaling cascade include the tyrosine kinase Btk which regulates membrane translocation and enzymatic activity of protein kinase CbetaI, which in turn is involved in transcriptional activation of IL-2 and TNF

genes through the c-Jun N-terminal kinase (JNK) pathway. Vav, a hematopoietic cell guanine nucleotide exchange factor, localizes to the plasma membrane in response to receptor aggregation and also regulates JNK. Once these pathways, important in regulating cytokine production and the inflammatory response, are dissected, insights into potential regulatory targets may emerge.

In exciting new observations, Branch scientists have demonstrated by gene targeting that secretory leukocyte protease inhibitor (SLPI) plays an important role in cutaneous wound healing. Absence of SLPI leads to delayed wound healing, an increased and prolonged inflammatory response, enhanced elastase activity, and delayed matrix accumulation. Thus, SLPI may play a number of surprisingly diverse roles in the wound healing cascade. In agreement with this proposed role of SLPI in wound healing, the application of exogenous SLPI to the aberrant wounds reduced the local inflammatory response and enhanced the rate of healing. The presence of SLPI in saliva and the perceived efficacy of licking of skin wounds may be a consequence of localized delivery of molecules such as SLPI, and may reflect an important evolutionary advantage with respect to the healing of wounds in nature. The generation of this animal model, reflecting age-related delayed wound healing in humans, makes it possible to experimentally approach and elucidate critical aspects of such patho-physiologic processes.

Unresolved immune activation is associated with autoimmune diseases such as Type 1 diabetes, also known as juvenile or insulin-dependent diabetes. IA–2 for which the genomic structure has recently been determined in OIIB and IA-2 beta are major autoantigens in Type 1 diabetes, and over 70% of patients have autoantibodies to IA-2. In contrast to Type 1 diabetes, Type 2 is not an autoimmune disease. In collaboration with colleagues in England and Germany, Branch scientists showed that about 5% of adult patients classified as Type 2 diabetics have autoantibodies to IA-2 and/or GAD. Re-classification to Type 1 is important because it will influence prognosis and therapeutic intervention. A further advance in our understanding of the pathogenic mechanisms in Type 1 diabetes revealed that the majority of IA-2 and GAD autoantibodies in diabetic and pre-diabetic subjects are of the IgG1 isotype, consistent with a chronic antigen-driven Th1 immune response both before and at clinical diagnosis.

In addition to innate and acquired immunity to self and foreign antigens, host defense may also encompass sensory mechanisms. Detection and discrimination between chemosensory signals underpins mammalian avoidance of toxic and noxious agents. In collaboration with scientists at UCSD, OIIB investigators isolated two families of G protein coupled taste receptors that are expressed in distinct subsets of taste receptor cells with distinctive topographic distribution. An additional T2R family of seven transmembrane taste receptors was identified which function as bitter receptors and are exclusively expressed in the G-protein, gustducin-positive cells of the tongue and palate taste buds. However, not all gustducin-positive cells in the front of the tongue co-express T2R, implicating additional receptor populations. In a remarkable achievement, a candidate murine bitter receptor, mT2R-5, was discovered, and the gene was mapped to the distal end of chromosome 6. Confirmatory evidence was obtained when a 5 amino acid mutation was identified in a nonresponsive (nontaster) mouse strain. The clustering of T2R and co-activation of the gustducin suggested a common innervation and therein, an explanation of why many structurally diverse toxins all taste bitter and engender an avoidance or protective response. These landmark studies were published in two *Cell* papers and highlighted on the *Cell* cover.

ORAL INFECTION AND IMMUNITY BRANCH
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Oral and Pharyngeal Cancer Branch

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ORAL AND PHARYNGEAL CANCER BRANCH

2000

Cancer of the head and neck area is the sixth most common neoplastic disease in the developed world, representing a very serious public health problem based on annual morbidity and mortality rates. The molecular and etiological factors involved in the development of head and neck tumors, including oral cancers, are still largely unknown. Members of our Branch work on complementary basic, translational, and clinical aspects of cancer research, in an effort to understand the molecular basis for malignant transformation as well as to use this knowledge to develop molecular markers of disease progression and novel therapeutic approaches for oral malignancies.

During the current reporting period, we have strategically initiated programs and recruited staff in areas that are relevant to head and neck cancer research. This includes the appointment of Dr. Adrian Senderowicz, Principal Investigator, to head a new unit focused on the development of novel "mechanism-based" therapeutic approaches for head and neck cancer patients, and the further expansion of Dr. Myung Hee Park, Dr. Frank Robey, Dr. Thomas Bugge, and Dr. Gutkind's research programs in areas of direct relevance to oral cancer research. This new direction is now affording us a better understanding of the basic mechanisms involved in squamous carcinogenesis and tumor metastasis, and provides an opportunity to investigate the still unknown molecular alterations that lead to oral malignancies. These efforts will likely broaden the horizon on developing potential oral tumor markers and treatments modalities. Furthermore, our Branch continued to play a leading role in a newly formed NCI/NIDCR/NIDCD Inter-Institute Consortium in Head and Neck Cancer, whose goal is the development of a comprehensive clinical research program in head and neck cancer at the NIH, based on the interdisciplinary expertise of the intra- and extramural components of the three institutes.

Our Branch has been highly productive during this year, and has made substantial contributions to the field, providing new concepts and shedding new light on questions of fundamental importance for cancer biology. We have also developed a large number of novel reagents, such as new genes, expression vectors, cell lines, antibodies, and bioactive peptides of value to biomedical research, and provided them to hundreds of investigators in the U.S. and abroad. Another tradition of our Branch has been our high priority on the training of postdoctoral investigators to become independent leaders in the field; we have tried to continue and strengthen this commitment.

During the current reporting period, significant progress has been made in a number of research efforts at the OPCB. A variety of arbitrarily selected research advances are highlighted below. The progress report for each project provides a more comprehensive description of the major findings in our Branch.

We have made a concerted effort to apply *state-of-the-art* genomic approaches to investigate the molecular basis of oral cancer. Utilizing clinical samples from patients with head and neck

squamous cell carcinomas (HNSCC), we demonstrated the successful use of laser capture microscopy (LCM) to procure specific cell populations, and that approximately 5,000 cells from representative tissue sets (tumor and normal from the same patient) were sufficient to extract RNA of high integrity for the construction of the first HNSCC-specific cDNA libraries. Initial analysis revealed that a large fraction of the cDNA clones represent novel sequences (22-44%), and the use of recently developed bioinformatic tools provided evidence for the existence of at least 200 novel genes in our cDNA libraries. Their putative role in the pathogenesis of HNSCC and/or their use as markers for early detection or as targets for pharmacological intervention in this disease can now begin to be evaluated. All relevant DNA sequences and clones are already available in the public domain. We have also used LCM to procure epithelial cells from a representative set of tumors and their matching normal tissues to explore the feasibility of establishing a pattern of expression of cancer-related genes in HNSCC by the generation of complex cDNA probes and DNA-chip screening methodologies. Using this approach, we found that HNSCC exhibit a distinctive pattern of expression of differentiation markers, signal transducing and cell cycle regulatory molecules, growth and angiogenic factors, and matrix degrading proteases. This work is expected to help identify gene products involved in the neoplastic process, as well as novel molecules representing clinically useful markers of oral carcinogenesis.

We have continued our drug evaluation effort at the NIDCR, whose goal is to develop novel therapies aimed at improving the quality of life and life expectancy of oral cancer patients. As part of this project, we have recently evaluated newly identified drug candidates for their effectiveness in squamous cell carcinomas as a collaborative effort with the NCI. One such drug candidate, flavopiridol, potently inhibited the proliferation of human squamous carcinoma cells and dramatically reduced the growth of tumor xenografts *in vivo*. The mechanism whereby this molecule affects cell growth has been extensively investigated. Furthermore, based on these preclinical results and the favorable outcome of the first Phase I trial of bolus flavopiridol in cancer patients, we now opened the accrual to the protocol "Phase II trial of daily bolus flavopiridol for five consecutive days in patients with recurrent/metastatic squamous cell carcinoma of the Head and Neck (SCCHN)", in collaboration with Medicine Branch, NCI, and the NIDCD.

The Kaposi's sarcoma associated herpesvirus (KSHV/ HHV 8) is implicated in the pathogenesis of acquired immunodeficiency syndrome-associated Kaposi's sarcoma (AIDS-KS), the most common malignancy in human HIV infection. The oral cavity is frequently involved in AIDS-KS and may represent one of the most frequent initial sites of this malignancy. As part of a collaborative effort, we have recently shown that a G protein-coupled receptor (GPCR) encoded by the ORF 74 of KSHV (KSHV-GPCR) displays constitutive activity, and is able to stimulate cell proliferation and expression of angiogenic growth factors, such as VEGF. Recently, we have elucidated the likely mechanism by which the KSHV-GPCR enhances the expression of VEGF. This involves the stimulation of the activity of the transcription factor HIF-1 alpha, which binds to and activates transcription from a hypoxia response element within the VEGF promoter. These findings provide the first insight into a mechanism whereby growth factors and oncogenes can interact with the hypoxia-dependent machinery of angiogenesis to stimulate VEGF expression.

Proteolytic modification of the extracellular matrix is essential for physiologic tissue remodeling, but also for the progression of a number of chronic, degenerative diseases including cancer invasion and metastasis. We have addressed the biochemistry, biology, and pathology of selected matrix degrading serine proteases. We are particularly interested in the plasminogen activation system, a complex system of serine proteases, protease inhibitors, and protease receptors, whose primary function is to govern the conversion of the abundant plasma protease zymogen, plasminogen (Plg), to the active protease, plasmin. The urokinase plasminogen activator receptor (uPAR) is a high affinity cell-surface receptor for the urokinase plasminogen activator (uPA). uPAR focuses uPA-mediated plasminogen activation to the cell surface, and may also have additional non-proteolytic functions including cell adhesion, migration, uPA internalization, and signal transduction. We are studying a novel transmembrane glycoprotein, termed the uPAR-associated protein (uPARAP), that associates with receptor-bound uPA. Interestingly, uPARAP may also be a specific high affinity cell surface receptor for the collagenase, matrix metalloprotease-13 (MMP-13), as well as for type V collagen. We studied the expression of uPARAP in mouse development, tissue homeostasis, and cancer. uPARAP mRNA was detected in trophoblast giant cells, the developing central nervous system, various embryonic and adult connective tissues, and, in particular, in the primary ossification sites of the developing long bones. Of interest, uPARAP mRNA was also strongly expressed in certain tumors including squamous carcinoma. We are now generating mice deficient in uPARAP to rigorously determine the functions of uPARAP in physiology and pathology.

We have investigated the growth and differentiation properties of normal human gingival keratinocytes (NHGK) and found that the [Ca^{++}] optimum for culture and the squamous envelope composition of oral keratinocytes are different from those of normal human skin keratinocytes. We found that when NHGK cells reached post-confluency in media containing 0.15 - 1.2 mM Ca^{++}, they undergo terminal differentiation in a similar fashion as *in vivo*. After culture in this medium for 3-5 days, induction of TGase 1 activity (5 to 10-fold increase) was followed by formation of insoluble cell envelopes (CEs) crosslinked by TGase 1. Indeed, TGase 1 is the key marker for terminal differentiation of oral keratinocytes. The mRNA levels of other markers of terminal differentiation, e.g. involucrin, SPR1 and annexin 1, were also increased in the Ca^{++}-induced NHGK cells. Furthermore, we obtained evidence that the cornified envelopes of NHGK cells contain an unusually high amount of SPR1, suggesting an important role of SPR1 in the specialized barrier function of oral epithelium.

Receptors coupled to heterotrimeric G proteins (GPCRs) can effectively stimulate growth-promoting pathways in a large variety of cell types, and if persistently activated, these receptors can also behave as dominant-acting oncoproteins. We have investigated the nature of the mitogenic and transforming pathways elicited by GPCRs as an experimental system for uncovering novel biochemical routes participating in the transduction of proliferative signals. Recently, we have focused on how this family of cell surface receptors induces the expression of the c-*jun* proto-oncogene. We have now found that GPCRs can elevate the activity of novel members of the MAP kinase family, including ERK1/2, JNKs, p38 alpha, p38 gamma, p38 delta, and ERK5, and that, in particular, the activation of ERK5 plays a central role in the regulation of c-*jun* expression. ERK5 exhibits an extended COOH-terminal tail, which is absent in other types of MAP kinases, thus suggesting that the regulation and function of this kinase might be different from that of other members of this family. We observed that receptors coupled to the G_q and

$G_{12/13}$ families of heterotrimeric G proteins, m1 and thrombin receptors, respectively, but not those coupled to G_i, such as m2 receptors, are able to regulate the activity of ERK5. Indeed, we found that the $Galpha_q$ and $Galpha_{12/13}$ families of heterotrimeric G proteins, but not $Galpha_i$, $Galpha_s$, and beta gamma subunits, are able to regulate ERK5. Furthermore, the use of dominant interfering molecules provided evidence that the stimulation of ERK5 by GPCRs involves a novel signaling pathway, which is distinct from those regulated by Ras and Rho GTPases.

ORAL AND PHARYNGEAL CANCER BRANCH
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Pain and Neurosensory Mechanisms Branch

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PAIN AND NEUROSENSORY MECHANISMS BRANCH
2000

The Pain and Neurosensory Mechanisms Branch (PNMB) conducts a multidisciplinary research program aimed at improved understanding and treatment of pain. Studies range from basic molecular and physiologic processes of nociceptive transmission, responses to tissue injury and peripheral inflammation (including subsequent plastic changes within the nervous system), to evaluating novel drugs and clinical hypotheses about pain and its control in human models of acute and chronic pain. The hallmark of the Branch's research program is the integration of basic, translational and clinical research which permits not only a rapid transfer of new findings from the laboratory to the clinic, but also fosters basic research on clinical problems. This integrative approach provides an optimal environment for training clinicians and basic researchers in the principles and methods of pain research across a spectrum spanning basic molecular mechanisms to the clinical management of pain. In addition, the Branch's senior investigators contribute nationally and internationally to the transfer of emerging scientific information to the training of clinicians and the treatment of patients by speaking, writing, and collaborations with professional organizations, academic institutions, and patient advocacy groups.

The independence and challenges presented by the NIDCR reorganization and the opportunities for PI-initiated research have increased scientific vigor and productivity in the Branch. The Branch continues to operate a large clinical research program, the Pain Research Clinic, in the Magnuson Ambulatory Clinical Research Facility under the scientific direction of Drs. Max and Dionne. Research conducted in this clinic is often based on observations made in the Branch's basic laboratories as well as using novel and prototypic drugs to test emerging scientific hypotheses in man, representing a true 'molecules to man' continuum. Dr. Gracely conducts collaborative research with the Department of Psychiatry, Harvard University and The Chronic Pain and Fatigue and Research Center at Georgetown University using functional MRI to evaluate basic pain mechanisms in healthy control and in patients with fibromyalgia. Drs. Dionne and Max have each edited texts on pain and symptom management, respectively, for publication in 2001. Publications over the past year in respected peer-reviewed scientific journals attest to the Branch's continued scientific impact. Highlights of research findings by Branch investigators during the past year are presented below.

Gene discovery program for persistent pain states: Scientists in the Neuronal Gene Expression Unit (NGEU) are investigating genes that are up-regulated in persistent pain states to better understand the molecular mechanisms underlying the transition to pain chronicity. Initial observations suggest that membrane remodeling occurs with pain input involving a GTPase signaling pathway in the spinal cord and that a secreted proteinase, cystatin C, is induced. This information is the basis for a clinical protocol being done in collaboration with an anesthesiologist at the University of Pennsylvania to examine cerebrospinal fluid levels in pain patients. Parallel studies are using in situ hybidization for cellular localization to individual spinal laminae of the novel genes that have been identified. Future plans are to localize the cells in the spinal cord that express the novel genes, compare pain to no pain conditions, make

libraries from ganglia of the cranial nerves innervating structures in the head and craniofacial structures to search for therapeutic targets for analgesia.

Blocking pain transduction at the vanilloid receptor: One of the most important molecules in pain transduction, the first step in pain sensation, is the vanilloid receptor 1 (VR1) which transduces the physical (heat) and chemical activation of receptors/ion channels in nociceptive primary afferent nerve endings located on skin and in deep tissues. In response to activation, nociceptors often release inflammatory substances which further enhance the pain signal, leading to sensitization of the peripheral nerve terminal. Investigators in the NGEU have tagged the VR1 C-terminal end with green fluorescent protein and subjected the molecule to truncation and point mutation to expedite molecular characterization. This approach has dissected the heat sensing motif from that of the chemosensor motif, and identified the residue that is necessary for potent vanilloid binding and functional activation of the receptor. These data have identified the region of VR1 that is critical for vanilloid-induced signal transduction and physical channel opening in response to painful temperatures. In addition, *in vivo* cellular imaging of calcium flux and organelles (endoplasmic reticulum and mitochondria) have identified new mechanisms of calcium toxicity. Rapid cellular imaging after vanilloid administration has identified massive alterations of cell structure that occur in the first few seconds of calcium toxicity leading to rapid cell death. Delineation of this process provides the scientific basis for a new cell deletion approach to treat chronic painful conditions in humans (described below).

Neonatal persistent pain produces plasticity in nociceptive neuronal circuits: Plasticity in the nervous system is increasingly recognized as a potential mechanism of chronic pain which persists after the initial stimulus or injury has been removed. Work in the Cellular Neuroscience Section focuses on the development of pain pathways and the effect of persistent pain and tissue injury during early neonatal development. An animal model of hind paw peripheral inflammation has been used to investigate the development of spinal cord circuitry in animals that experienced persistent inflammatory pain as neonates. Behavioral responses to a test of acute and tonic pain sensitivity, the formalin test, were altered in adult rats treated neonatally with an inflammatory agent. These rats exhibited higher evoked firing rates in response to brush and noxious pinch relative to the same intensities of stimulation applied to untreated rats. These data demonstrate a dramatic alteration in spinal cord neuronal circuitry after persistent neonatal inflammatory pain. This plasiticity in nociceptive neuronal circuits in the adult may explain unique developmental and behavioral differences in neonates who experience persistent pain and highlights the need for adequate pain management in the neonate.

Dorsal root mechansims of antinociception: Scientists in the Clinical Measurement and Mechanism Unit (CMMU) use novel methods to evaluate peripheral mechanisms of nociception. This year they discovered the fastest know conduction velocities in nervous system of the rat, a mean of 221 m/s for antidromic conduction in the dorsal root, considerably faster than previous fast speeds of approximately 120 m/s. The role of these fast velocities was investigated in a study of descending inhibition, which showed that analgesia mediated by central brain sites such as the periaqueductal gray (PAG) is likely due in part to fast activity transmitted the "wrong way" out primary afferents, resulting in peripheral collision with primary afferent activity.

Genetics of chronic neuropathic pain: The Clinical Trials Unit initiated a program this past year to evaluate the genetic mechanisms of chronic neuropathic pain in collaboration with the Molecular Epidemiology Branch. Based on previous studies in a rodent model demonstrating recessive transmission of a trait for developing neuropathic pain after sciatic nerve injury, the intensity of pain behavior was studied in different inbred strains of mice. One parenteral strain was identified with high pain behavior and one with minimal pain behavior and 26 recombinant strains derived from a cross of these strains were evaluated for pain behavior in the sciatic nerve model. The recombinant strains varied widely in levels of pain behavior and resultant analysis by Dr. Scott Diehl's lab resulted in localization of the trait to a small part of one chromosome containing several promising candidate genes. Future studies will more finely localize the responsible genes.

Selective neurotoxins and gene transfer treatments for chronic pain: Adequate control of cancer pain remains a significant clinical problem. To reduce side effects of treatment, spinal routes of administration have been used to achieve regional pain control with a reduced drug dose. The NGEU has develop three investigational strategies for pain therapy by directly translating basic science findings to create new treatments for chronic pain. The first is the use of vanilloids to delete pain-sensing cells from the dorsal root ganglion by direct intra-ganglionic injections. This approach can be used in humans by direct gangionic injections coupled with CT or MR imaging methods. The second approach employs *in vivo* gene transfer to direct extracellular release of neuroactive peptides from cells surrounding the spinal cord. The third approach uses cell deletion of second-order neurons in the spinal cord by administering a substance P-Pseudomonas exotoxin ligand. This approach has been demonstrated to block thermal and mechanical pain as well as inflammatory hyperalgesia. All three of these strategies are now undergoing preclinical toxicology studies as a prelude to clinical trials in patients with intractable cancer pain.

Evaluation of COX-2 selectivity at the site of tissue injury: The analgesic effect of selective cyclooxygenase-2 (COX-2) inhibitors is likely mediated through COX-2 expressed following tissue injury. The expression of COX-2 was demonstrated in tissue biopsies collected at the onset of acute pain following oral surgery, supporting an involvement of COX-2 in the early phase of inflammation in the oral surgery model. Evaluation of the role of COX-2 in producing pain following tissue injury was further evaluated by measuring local levels of prostaglandin E_2 – a product of both COX-1 and COX-2 – and thromboxane B_2 – a measure of COX-1 activity – using microdialysis probes placed under the surgical flap. The temporal profiles of these mediators following surgery and during pain onset was consistent with constitutive COX-1 activity and inducible COX-2 activity. Comparison of the selective COX-2 inhibitor celecoxib to the dual COX-1/COX-2 inhibitor ibuprofen suggests that celecoxib is less selective for inhibition of COX-1 *in vivo* than preclinical *in vitro* and *ex vivo* studies had indicated. These studies permit assessment of mechanistic hypotheses of drug actions that are formulated in animal models and *in vitro* but not otherwise subject to confirmation in humans.

Spatial and temporal cerebral patterns evoked by punctate and blunt pressure pain: Tenderness to blunt pressure is the presenting clinical symptom in diseases such as fibromyalgia. The CMMU has characterized the cerebral response to fluctuating and constant blunt pressure applied to the thumb. This painful stimulus activates a network related to the side stimulated,

and also specific responses in the left thalamus and right prefrontal cortex regardless of the side simulated. The response to constant pressure included an additional bilateral activation of orbital cortex, and the overall response showed 3 distinct patterns of activation in a 15-second period. These results challenge the concept of a unitary pain circuit, demonstrating unique networks over qualitatively different conditions and over brief periods of time. An additional study evaluates the effects of intense, brief punctate mechanical stimuli in a high-field 3T MRI scanner. Preliminary evidence demonstrates activation of a pain network in individual subjects after only 24 total seconds of painful stimulation. This promising method will be applied to new studies to individual differences in spatial and temporal responses to acute pressure and to the effects of potent opioid analgesic agents.

These highlights of the studies summarized in the individual reports support the rationale and importance of conducting translational studies in humans to evaluate mechanisms of pain and analgesic strategies based on studies of nociceptive mechanisms in animal models and in vitro assays. Future studies by investigators in the Branch will extend these novel analgesic mechanisms to block the molecular-genetic processes leading to the development of pain chronicity following tissue injury and to evaluate the clinical utility of cell deletion and gene transfer as chronic pain treatments.

PAIN AND NEUROSENSORY MECHANISMS BRANCH 2000 BIBLIOGRAPHY

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Functional Genomics Unit

Ashok Kulkarni

FUNCTIONAL GENOMICS UNIT
2000

Our Unit continues to make exciting research advances in the pursuit of basic scientific knowledge in dissecting pathways relevant to the disorders that affect craniofacial and dental systems because of genetic abnormalities. Our primary research approach is centered on functional genomics and the key research areas are concentrated on the following studies:

In the first set of studies, we have begun to delineate precise roles of Cyclin dependent kinase-5 (Cdk5) in neuronal phosphorylation to gain insight into its role in abnormal phosphorylation observed in a number of neurodegenerative disorders. Following the cloning of the Cdk5 gene, we generated mice deficient in Cdk5 expression which exhibit perinatal mortality associated with gross lesions in the brain and spinal cord. These mice lack normal stratification of the neurons along with cerebellar defoliation, accumulation of neurofilament-H in the neuronal cell bodies and ballooned motor neurons. Further studies revealed a typical inverted cortex in these mice indicating a special "cell autonomous" role of Cdk5 in neuronal migration. We have now reconstituted Cdk5 expression, mainly in the nervous system of the Cdk5 null mice by transgenic technique, which indicates that its neuronal expression is critical for survival. Additionally, we have generated Cdk5 "conditional" knockouts that mimic an ALS-like phenotype, suggesting peripheral neuropathy to reverse metabolic defects in Fabry mice. Since other lipidosis disorders exhibit dental abnormality, we have carried out dental examination of Fabry patients and also of Fabry mice that reveal interesting malocclusions in the oral structures.

In the second project, we chose to work on Fabry disorder because of its unique nature as a painful and fatal metabolic disorder and the challenges it presents in developing much needed therapeutic approaches. Following the cloning of the murine gene α-galactosidase A, the gene involved in Fabry disease, we generated null mice which exhibit lipid inclusions in the target organs typically seen in Fabry patients. Subsequent studies revealed that the aging of these mice accentuates and bone marrow transplantation ameliorates the phenotype of these mice, indicating a potential for BMT as a therapy for some Fabry patients. In collaboration with others, we have also demonstrated effectiveness of other therapeutic approaches such as gene therapy and substrate deprivation.

In the third set of projects, we have analyzed the autocrine and endocrine roles of transforming growth factor -ß1 (TGF-ß1) in inflammation as well as in tooth development. The initial findings from these studies indicate ameliorating effects of MHC-1 deficiency in inflammatory responses in the absence of TGF-ß, potential for de novo TGF-ß1 gene therapy and also involvement of TGF-ß1 in tooth mineralization. Interestingly, mice overexpressing TGF-ß1 in teeth mimic tooth abnormalities seen in dentinogenesis imperfecta and dentin dysplasia.

In the fourth project, we have initiated studies on molecular genetics of tooth development and disease. We have begun to delineate the in vivo role of the dentin sialophosphoprotein gene (dspp)and the amelogenin genes in dentinogenesis and amelogenesis, respectively. We have cloned and partially characterized the genomic structures and expression patterns of these genes.

We have developed a transgenic animal model with a reporter gene (ß-galactosidase) under the control of the 5.7 kb

5'- sequence flanking the dspp gene and the analysis of these mice validates the dspp promoter for tooth specific expression of the candidate genes and Cre transgene. Additionally, we have disrupted the genomic loci of both the genes in ES cells to generate gene knockout mouse models for DGI-II and AI, respectively.

FUNCTIONAL GENOMICS UNIT
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Immunopathology Section

Larry Wahl

comparative studies between LPS and the cytokines on the signaling pathways have demonstrated that they utilized different MAPKs to regulate MMP-1. LPS stimulates MMP-1 synthesis primarily through p38 whereas the combination of TNF alpha and GM-CSF induce MMP-1 through ERK1/2. However, ERK1/2 is the predominate pathway utilized by both LPS and the cytokines in the induction of MMP-9.

In collaboration with researchers at the CDC we have examined the effect of *Mycobacterium avium* (*M. avium*) on replication of human immunodeficiency virus type 1 (HIV-1) in resting T cells and the potential role of MMP-9 in modulating this process. Two human clinical isolates (serotypes 1 and 4), but not an environmental *M. avium* isolate (serotype 2), enhanced HIV-1 replication. The *M. avium*-induced HIV-1 replication was not associated with cell activation or differential cytokine production or utilization. Addition of MMP inhibitors, either chemical compounds or the natural inhibitors, TIMP-1 or TIMP-2, abrogated *M. avium*-induced HIV-1 replication by 80-90%. The MMP inhibitors did not have any effect on the HIV-1 protease activity, suggesting that they may affect cellular processes. Furthermore, MMP-9 protein was differentially expressed after infection with clinical *M. avium* isolates and paralleled HIV-1 p24 production. Collectively, these data suggest that *M. avium*-induced HIV-1 replication is mediated, in part, through the induction of MMP-9.

IMMUNOPATHOLOGY SECTION
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Matrix Metalloproteinase Unit

Henning Birkedal-Hansen

MATRIX METALLOPROTEINASE UNIT
2000

The Matrix Metalloproteinase (MMP) Unit seeks to define the role of MMPs and their inhibitors in physiologic and pathologic processes. Recently, we have focused particular attention on membrane-bound metalloproteinases and their dual role in degrading the extracellular matrix and activating other members of the MMP family.

Targeting of the MT1-MMP Gene

We have shown earlier that disruption of the gene of the membrane-bound metalloproteinase, MT1-MMP, results in a plethora of growth-related abnormalities in both skeletal and soft connective tissues. This is by far the most severe phenotype generated by targeting of any MMP or MMP inhibitor. Our findings showed that MT-MMP plays a critical role in post-embryonic growth and development whereas early embryonic development is less severely impaired. We were able to causally link the phenotypic defects including growth retardation, skeletal deformities, craniofacial defects, skin fibrosis, dwarfing and arthritis-like joint abnormalities to inadequate degration and remodeling of collagenous matrices. During the past year we have pursued a number of questions raised by this phenotype including a more detailed analysis of the growth-plates from knock-out and control animals. These studies were conducted in collaboration with Robin Poole's laboratory in Montreal. Our findings showed that in spite of severe growth retardation and dwarfing, the growth plates go through a normal and essentially correctly timed developmental sequence including hypertrophy, resorption and bone formation for the first 40 days or so. Later on, the growth plates become increasingly dysfunctional and essentially shut down activity by day sixty. These observations suggest that growth plate abnormalities are not a primary defect in these animals but come into play rather late in the already shortened lifespan of these animals.

Targeting of Two other Membrane-Associated Matrix Metalloproteinases

Structural studies have suggested that MMP-19 and MT4-MMP (MMP-17) most likely are linked to the plasma membrane through GPI-anchors. They therefore represent a new and unique subgroup of MMPs. To further explore the function of these enzymes, we targeted each of these genes for disruption in mice; MMP-19, in collaboration with Dr. Carlos Lopez-Otin, Oviedo, Spain. These efforts appear to have been successful and the phenotypes of homozygous animals are being analyzed.

Targeting of a Matrix Metalloproteinase Specific for Tooth Development

Previous studies have shown that enamelysin (MMP-20) is expressed only by odontoblasts and ameloblasts, and only during certain stages of tooth development. These features make MMP-20 an attractive candidate for gene blastion strategies in pursuit of biologic function. The homozygous MMP-20 -/- somewhat surprisingly appear to display no tooth-related phenotypic abnormalities. Tooth formation is not delayed nor does the histology, eruption or function of incisor and molar teeth appear to be altered. While additional phenotypic studies are being conducted in collaboration with Dr. John Bartlett, Forsyth Research Institute, Boston, our findings have led to the tentative but surprising conclusion that enamelysin is not critical for normal odontogenesis.

Targeting of the TIMP-2 Gene

A number of studies have suggested that TIMP-2 plays a critical role in physiology and pathology for several reasons: (i) TIMP-2 (unlike TIMP-1) selectively inhibits membrane-type metalloproteinases, and (ii) TIMP-2 participates in formation of a ternary complex with MT1-MMP and gelatinase A (MMP-2) which appears to be important for physiologic activation of gelatinase A. We therefore targeted the TIMP-2 gene for disruption and produced homozygous mutant mice which still express low levels of mutant protein but have lost >99% of TIMP-2 inhibitory activity.

Disruption of the TIMP-2 gene gave rise to seemingly minor phenotypic changes in growth and development yet cells derived from mutant mice showed markedly reduced ability to activate gelatinase A both *in vivo* and *in vitro*. Notably, the TIMP-2 mutant mice failed to match the dramatic phenotypic changes of the MT1-MMP -/- mouse suggesting that gelatinase A activation (by means of TIMP-2 bridging) does not play an important role in growth and development. Further analysis, however, unveiled significant and profound differences between wildtype and mutant mice in a murine tumor model in which the TIMP-2 mutant genotype was bred into a transgenic mice carrying middle T antigen gene under control of the mouse mammary tumor virus promoter. Primary breast tumors develop in virgin females by 2 months of age which then, by a random genetic event, metastasize to the lungs at high frequency by 4 months of age. In this model, mice homozygous for the mutant TIMP-2 locus had much lower levels of lung metastasis than those with wildtype TIMP-2 loci. We also noticed that matrix-associated gelatinase A is activated in extracts of normal lungs, but remains latent in extracts of lungs from TIMP-2 deficient mice, providing evidence for a role of TIMP-2 in regulation of progelatinase A activation *in vivo*. While the mechanism remains to be understood in greater detail, these findings suggest that activation of progelatinase A may play a significant role in regulating mammary tumor metastasis.

Regulation and Activation of Collagen Degradation

In search of enzymes and inhibitors which regulate and catalyze the metabolic degradation of extracellular matrix, we pursued a strategy which allows us to monitor the dissolution of a collagen type I fibrils by live cells. Transfection (reconstitution) experiments performed with COS and CHO cells (which do not normally degrade collagen fibrils) permitted us to define the minimal requirements for generation of a collagenolytic phenotype. These experiments showed that furin, MT1-MMP and either gelatinase A *or* collagenase-3 are minimally required and/or rate limiting. Since MT1-MMP contains a furin cleavable activation site, we surmise that the sequence of events includes (but may not be limited to) furin activation of MT1-MMP followed by MT1-MMP activation of gelatinase A or collagenase-3. Both gelatinase A and collagenase-3 are capable of cleaving reconstituted fibrils of type I collagen.

MATRIX METALLOPROTEINASE UNIT
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Molecular Structural Biology Unit

Dennis Torchia

MOLECULAR STRUCTURAL BIOLOGY UNIT
2000

The principal research goal of the MSBU is to elucidate the structure and dynamics of proteins and associated molecules at the molecular level in order to provide a basis for understanding function. The main research tool used in this work is high resolution, multidimensional, nuclear magnetic resonance (NMR) spectroscopy. Three projects are currently active (1) HIV-1 protease, as either the free enzyme or bound to a high affinity protease inhibitor (2) Ribosomal proteins S4 and L11, either free or bound to a target RNA molecule (3) VIAF, a protein that regulates apoptosis. A fourth project, on the ribosomal inactivating protein MAP-30, was concluded during the first half of the reporting period. Progress that has been made during the past year, relating the structure and dynamics of these molecules with their functions is discussed below.

HIV-1 protease

Potent inhibitors of the HIV-1 protease currently used in therapies against AIDS have a limited effect due to selection of drug-resistant variants of the virus. Hence, understanding inhibitor-protease interactions at the molecular level, remains of great interest. X-ray diffraction provided early information about the interactions between the protease and inhibitors. These studies, together with recent NMR studies of the protease in solution, indicate that dynamics of the protease plays an important role in protease/inhibitor interactions. NMR relaxation measurements provide information about internal motions of proteins on fast (sub-nanosecond timescale) and slow (μs-ms) motions in proteins. Our initial work focused on studies of backbone dynamics of the free protease and the protein bound to the potent inhibitor, DMP323. These studies showed that in the free protease (but not in the bound protease) both fast and slow internal motions take place for NH bonds in flap residues G48 through F53 (the flaps cover the substrate binding site, and when open, permit access to the active site). We hypothesized that the fast motions of the flaps involve fluctuations among the semi-open beta-hairpin conformations, whereas the slow motions reflect the dynamic equilibrium between the semi-open and fully open flap conformations, that permit entry of substrates and inhibitors to the active site. These studies also revealed flexibility of the interfacial beta-sheet that stabilizes the protease dimer structure. Flexibility of the interface may enhance protease auto processing by making the protease-RT junction accessible for cleavage. Recently we have begun to study the flexibility of protease sidechains. Methyl group flexibility is of particular interest because nearly 50% of the amino acid residues of HIV-1 protease contain methyl sidechains, most of which appear to be organized in inner (I)- and outer (O)-clusters. Residues in the I-cluster make up part of the inhibitor binding site, while those in the O-cluster form the hydrophobic core that stabilizes the protein structure. In addition nearly 2/3 of the mutations associated with drug resistance are in methyl containing residues. Using a novel labeling approach that enabled us to record both 13C and 2H relaxation data we found that many methyl sidechains, in both free and DMP323 bound protease were flexible on fast and slow timescales. Flexible methyl sites, that are partially or fully buried, were found in both methyl clusters as well as in residues that link the clusters. These flexible sites, particularly in the residues that link the clusters, may allow the protease to adjust its conformation in response to the binding of a variety of substrates and to mutations in its amino acid sequence that are selected by drug-treatment. Because the methyl cluster motif appears to

be a common structural feature of retroviral proteases, it may play a similar role throughout this family of enzymes. While reasonable, these hypotheses need to be tested by studies of spin relaxation, kinetic parameters and structural stability of mutant proteases selected for drug resistance.

Structures of the ribosomal proteins S4delta41, S4 and L11.

Prokaryotic ribosomal protein S4 binds to 16S rRNA to nucleate assembly of the small subunit of the ribosome. Mutations in S4 affect translational accuracy and antibiotic resistance. S4 also binds to its mRNA to regulate its own translation. The RNA targets for S4 have been reduced to disparate structures, the mRNA site includes about 100 nucleotides in a pseudoknot, while the rRNA site includes about 460 nucleotides in hairpins. As a step toward understanding how S4 recognizes such disparate targets, we first solved the structure of its mRNA binding domain (S4delta41, 159 residues) by solution NMR spectroscopy. S4delta41 has a novel fold with two distinct subdomains. The cleft between the subdomains is lined with positively charged side chains from both subdomains, suggesting a likely RNA-binding site. We examined the binding of S4delta41 to 112 nucleotides from the mRNA. By mapping changes in the ^{15}N chemical shifts onto the corresponding residues in the protein, we recognized that the RNA binding site coincides with the positively charged face of the protein. However, broadening of certain peaks suggests that the protein-RNA complex is dynamic on the time scale of the measurements. Moreover, band shift assays showed that nonspecific binding occurs under our sample conditions. We have probed pH, buffer, salt concentration, and temperature, but so far, we have not identified conditions which abolish non-specific binding. Dipolar couplings were crucial for determining the relative orientation of the subdomains of S4delta41. These measurements require slight alignment of the protein, induced by a liquid crystalline medium. Of the media currently in use, S4delta41 seems to disrupt formation of a stable phase with bicelles and interacts with the Pf1 phage, possibly disturbing S4delta41's conformational equilibrium. We are exploring acrylamide gels as a medium for dipolar measurements, in collaboration with Robert Tycko in the NIDDK. Intact S4 binds to 16S rRNA about ca. 50 fold better than S4delta41. We have recently shown that the structure of the C-terminal 158 residues of the intact protein is the same that of S4delta41, while the 45 N-terminal residues are very flexible. The structure these residues was modeled from using secondary chemical shifts and structural restraints derived from NOESY data. The results of these studies indicated that two regions of the N-terminus, $S_{12}RRL_{15}$ and $P_{30}YPP_{33}$, adopt transiently ordered structures. Transient order was revealed by relatively small RMSDs (calculated using XPLOR) of phi and psi dihedral angles and of the positions of backbone atoms. In addition, ^{15}N relaxation data showed relatively small T_2 values and relatively large heteronuclear NOE values for these regions, suggesting that their motion is more restricted than the remainder of the N-terminus. A BLAST search revealed that R13, R14, P33, G34, and H36 are completely conserved in S4 molecules from both eubacterial and chloroplast ribosomes of at least 27 species. Careful analysis of the modeled structures of $S_{12}RRL_{15}$ indicated that the backbone adopts a bent conformation, which directs the two conserved arginine side chains out into solvent in a parallel fashion, suggesting a possible RNA-binding site. Similar analysis of the structures of $P_{30}YPP_{33}$ indicated that these residues form a nascent turn of a polyproline II helix, a common motif involved in protein-protein interactions. As a whole, the data suggest a model in which the N-terminus of S4 is in general flexible and disordered, but which nevertheless contains two conserved segments, $S_{12}RRL_{15}$ and $P_{30}YPP_{33}$, that may be respectively important in the interaction of S4 with RNA and other

ribosomal proteins in the intact ribosome. L11 is a protein component of the large ribosomal subunit that is essential for efficient protein synthesis. We previously determined the solution structure of the C-terminal portion of L11 and have now obtained a high resolution NMR spectrum of the complete molecule. After finding conditions that maximize the solubility of L11 we will determine the structure of the intact protein, free, and bound to a 58 nucleotide RNA target. A crystal structure of L11 bound to a 58 nucleotide rRNA target, shows high B factors for the N-terminal domain, and the N-terminal domain has not been located in the 2.4Å crystal structure of large subunit of the ribosome. The difficulty of placing the N-terminal domain may reflect L11's proposed role as a molecular switch. NMR is well-suited to determine if the linker between the N- and C-terminal domains is flexible, thereby allowing them to readily alter their relative orientation and act as a molecular switch.

Structure/function study of VIAF, a protein that regulates apoptosis

VIAFs are a conserved protein family (initially identified by our collaborators in Colin Duckett's lab in NCI), that associate with animal IAPs (inhibitor of apoptosis proteins). VIAF itself substantially protects cells from Fas- and Bax-induced apoptosis, while coexpression of VIAF with suboptimal quantities of XIAP conferred almost complete protection from these inducers. VIAF and XIAP activated JNK in a synergistic manner. Hence, VIAR is a novel cofactor which modulates the anti-apoptotic and signaling properties of the IAP family. Full length VIAF contains 239 residues, but because the C-terminal 128 residues are sufficient (and necessary) for interaction with IAPs from baculovirus, we have cloned a 158 residue (17.8 kDa) C-terminal construct of hVIAF (human VIAF) into a pET-16b vector which was then transformed into BL-21 cells. The His-tag protein was expressed in minimal media, purified using Ni-NTA column, treated with factor-Xa to remove the tag and subsequently passed over Ni-NTA and size exclusion columns. A one liter culture yields sufficient protein for NMR studies. The ^{15}N-HSQCspecturm of VIAF-C158 is well dispersed, indicating that it is a highly structured globular protein. The T_2 measured for VIAF-C158, 83ms is in reasonable agreement with that calculated for a 17.8 kDa protein at 298 K,105 ms. We have produced both 15N labeled and 15N/13C double-labeled material for a structure determination and are currently optimizing sample conditions for acquiring NMR spectra. When this is completed, we will determine the three dimensional solution structure of VIAF-C158. The interaction of VIAF-C158 with XIAP will also be studied by NMR; specifically, we will to map the amino acid residues of VIAF-C158 that interact with XIAP

Structure/function study of the anti-HIV/anti-tumor protein Map30 (project concluded)

MAP30 is a ribosome inactivating protein (RIP) derived from bitter melon. RIPs are of wide interest because they are cell toxins with potent anti-viral/anti-tumor activities. We have recently shown that the solution structure of MAP30 is similar to the crystal structures of other RIPs; hence, there is no obvious structural basis for reported activities unique to MAP30, such as inhibition of HIV integrase. Our hypothesis that this reported activity might reflect competition of MAP30 and integrase for a common DNA substrate was supported by (1) chemical shift perturbation data upon adding a target DNA and (2) the fact that $NaBH_4$ traps an imine cross-link between MAP30 and LTR RNA. These observations provide evidence that, after binding, MAP30 depurinates DNA and acts as DNA lyase. Further biochemical studies showed that MAP30 specifically depurnated adenine sites in DNA targets. Based upon the biochemical data and detailed structural analysis we proposed that RIPs utilize a common active site to depurinate

both DNA and 28SrRNA, and that, following depurination of DNA, the apurinic DNA site interacts with the neighboring conserved Trp sidechain, thus facilitating nucleophilic attack by a nearby Lys sidechain.

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